International Journal of Phytoremediation

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bijp20

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Available online: 15 Nov 2011

To cite this article: Zouhayr Arbib, Jesus Ruiz, Pablo Alvarez, Carmen Garrido, Jesus Barragan & Jose Antonio Perales (2012): Chlorella stigmatophora for Urban Wastewater Nutrient Removal and CO_2 Abatement, International Journal of Phytoremediation, 14:7, 714-725

To link to this article: http://dx.doi.org/10.1080/15226514.2011.619237

International Journal of Phytoremediation, 14:714–725, 2012

ISSN: 1522-6514 print / 1549-7879 online
DOI: 10.1080/15226514.2011.619237

CHLORELLA STIGMATOPHORA FOR URBAN WASTEWATER NUTRIENT REMOVAL AND CO_2 ABATEMENT

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***Batch experiments were performed to study biomass growth rate, nutrient removal and carbon dioxide bio-fixation of the marine microalgae* Chlorella stigmatophora*. Four different cultures at different salinities were tested: wastewater (WW), synthetic wastewater (SWW), seawater (SW) and diluted seawater (DSW). Experimental results showed that* Chlorella stigmatophora *grew satisfactorily in all culture media, except in SWW where inhibition occurred. In all cases, biomass experimental data were fitted to the Verlhust Logistic model ($R^2 > 0.982$, $p \leq 0.05$). Maximum biomass productivity (P_{bmax}) and CO_2 biofixation (P_{vCO2}) were reached in the WW medium, 1.146 g SS·L^{-1}·day^{-1} and 2.324 g CO_2·L^{-1}·day^{-1} respectively. The order of maximum specific growth rates (μ_{max}) was WW >DSW>SW. In order to compare nitrogen and phosphorous removal kinetics, an estimation of the time required to reach the most restrictive concentration of total N and P in effluents as defined in the Directive 98/1565/CE (10 mg $\Sigma N \cdot L^{-1}$ ($T_{10(N)}$) and 1 mg $\Sigma P \cdot L^{-1}$ ($T_{1(P)}$) was performed. In the WW test $T_{10(N)}$ and $T_{1(P)}$ needed were of 45.15 and 32.27 hours respectively and at the end of the experimental the removal was in both 100%.*

KEY WORDS: microalgae, wastewater, nutrients, carbon dioxide, *Chlorella stigmatophora*

INTRODUCTION

The increase of urban wastewater production associated to the exponential population growth rate is one of environmental challenges in the world. These effluents have to be treated to reduce contaminants concentrations to environmentally safe levels before discharging the wastewater into rivers, lakes, or the sea. Special attention is required for inorganic substances such as ammonium, nitrates and phosphates, which may contribute to the eutrophication of the water bodies receiving these effluents. Numerous studies have focused on nitrogen and phosphorus removal from wastewater. Most of the studies are based on biological processes and different combinations of anaerobic, aerobic, and anoxic zones

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such as Bardenpho, A2O, UCT (University of Cape Town process), and their modifications. Sequencing batch reactor (SBR) operations have been used by many investigators for nutrient removal purposes (Kargi and Uygur 2003; Obaja *et al*. 2005)

Actually, both biological and physicochemical treatment technologies for nutrient removal have several disadvantages such as sludge production, operation and infrastructure costs. One of the factors that contribute to efficient and reliable nitrogen removal is an adequate supply of a carbon source (internal or external) (EPA 2008).

Wastewater treatment by microalgae has great advantages over conventional treatments: (1) nitrogen and phosphorous can be converted into biomass without any external organic carbon source (2) discharge of oxygenated effluent into the water bodies, and (3) high value products can be extracted from generated biomass. The great disadvantage was the recovery of microalgae biomass because of the small size (3–30 mm diameter) of the algal cells, Recovery of the biomass from the broth has been claimed to contribute 20–30% to the total cost of producing the biomass (Gudin and Therpenier 1986).

Many studies have showed that microalgae have a great potential for nitrogen and phosphorus removal (De la Noue and De Pauw 1988; Tredici *et al*. 1992; Oswald 1995; Gonzalez *et al*. 1997; Mallick 2002). The main mechanism for algal nutrient removal from wastewater includes uptake into the cell and ammonia stripping through elevated pH (Hoffman 1998; Bich *et al*. 1999). The cultivation of algae in wastewater offers the combined advantages of nutrient removal and biomass production, as a potential source of proteins (Kuhad *et al*. 1997), carbohydrates (Martínez *et al*. 2000), pigments (Wilshire *et al*. 2000), lipids (Isik *et al*. 1999), and hydrocarbons (Kalacheva *et al*. 2002). In addition, the biomass can be used as a low releasing fertilizer (Mallick 2002).

Another important characteristic of microalgae is that they have a great potential for carbon dioxide capture (Borowitzka 1999; Chisti 2007; De Morais and Costa 2007a; Li *et al*. 2008). Therefore carbon dioxide can be incorporated into the molecular structure of cells as proteins, carbohydrates and lipids by way of photosynthetic reactions. Coupling the cultivation of photosynthetic microorganisms in wastewater with the biofixation of carbon dioxide has the potential not only to reduce the costs of culture media for growing such organisms on an industrial scale but also to offset carbon emissions (Beneman and Hughes 1997).

The aim of this work is to study the possibility of culturing the green marine microalgae *Chlorella stigmatophora* in urban wastewater, by evaluating the effect of the salinity in the biomass production. Nutrient removal rate and carbon dioxide fixation of that marine microalgae has also been investigated.

MATERIALS AND METHODS

Microorganism

C. stigmatophora was purchased from the Laboratory of Marine Culture at the Marine and Environmental Sciences Faculty of the University of Cádiz. The monoculture were grown in sterilized seawater, the sterilization was done with ultraviolet radiation, enriched with synthetic medium F/2 (Guillard and Ryther 1962) (Table 1) Inocula for the experiments were incubated at 20 $\pm$ 1°C and 250 μmole cm^{-2} s^{-1} light intensity under 14:10 h light dark cycle.

Table 1 Composition of synthetic medium F/2 (Guillard and Ryther 1962)

Components	Concentration (mg·L-1)
NaNO3	75
NaH2PO4·2H2O	5.65
Na2·EDTA	4.16
FeCl3·6H2O	3.15
CuSO4·5H2O	0.01
ZnSO4·7H2O	0.022
CoCl2·6H2O	0.01
MnCl2·4H2O	0.18
Na2MoO4·2H2O	0.006
Vitamin B12	0.0005
Vitamin B1	0.1
Biotin	0.0005

Test Culture Media

Four different culture medium were tested in this project (Table 2). Firstly the pretreated wastewater (WW) (1200 μS·cm^{-1}) was taken from the effluent of an urban wastewater treatment plant located in the city Arcos de la Frontera (South of Spain). The sample was filtered by 1 μm glass fiber filter and autoclaved for 20 min at 1 kg·cm^{-2} to assure monospecific cultures of the microalgae. During the sterilization process salt precipitation was observed, so a vigorous stirring was needed to resuspend the precipitated salts.

The second culture medium was a synthetic wastewater (SWW) (50 μS·cm^{-1}), this was composed of autoclaved bidistilled water (miliQ quality) enriched with a modified F/2 medium to contain about 3 mg·L^{-1} P-PO_4 as K_2HPO_4, 0.3 mg·L^{-1} N-NO_3 as $NaNO_3$ and 20 mg·L^{-1} N-NH_4 as NH_4Cl. These concentrations were added to fit the urban wastewater effluent from Arcos de la Frontera (Spain) treatment plant.

The third media tested was sterilized seawater, the sterilization was made by Ultraviolet radiation (SW) (54000 μS·cm^{-1}), sampled in a non-polluted nearby coastal area (36°31′54.70″N; 6°12′54.62″W). The SW was enriched with N-NO_3^- as $NaNO_3$ and P-PO_4 as K_2HPO_4 to obtain a composition similar to WW.

Table 2 Composition of the different culture media tested. WW(wastewater) SWW(synthetic wastewater); SW(sea water); DSW (diluted sea water)

Culture medium	N-NH4+ (mg.L-1)	N-NO3- (mg.L-1)	[b]ΣN	[c]R_N	P-PO43- (mg.L-1)	[d]N/P	Conductivity (μS.cm-1)
WW	20.45	0.352	20.80	58.18	3.34	6.2	1200
SWW	19.67	0.352	20.02	55.89	2.99	6.7	50
SW	0.157	23.08	23.24	0.006	3.65	6.4	54000
DSW	0.175	22.34	22.51	0.008	3.50	6.4	1200
[a]CTR	0.117	19.61	19.73	0.006	3.81	5.2	50

[a]CTR: Control reactor. Not inoculated with microalga.
[b]ΣN: N-NH_4^+ + N-NO_3^-.
[c]R_N: N-NH_4^+/N-NO_3^-.
[d]N/P: ΣN/P-PO_4^{3-}.

Finally a diluted sterilized seawater (DSW) (1200 $\mu S \cdot cm^{-1}$), also enriched with N-NO_3 as $NaNO_3$ and P-PO_4 as K_2HPO_4. The dilution was made with bidistilled water (MiliQ quality) to obtain a salinity similar to the WW (1200 $\mu S \cdot cm^{-1}$).

Experimental Design

Tests were conducted in bubble columns PBRs, made of transparent polymethyl methacrylatate (PMMA) with 3.3 mm thick, 72 mm internal diameter, and 700 mm height. PBRs columns were previously sterilized using Peracetic acid 5mM during 30 minute. The fluid was mixed and aerated with 0.2 μm prefiltered air. The air stream was bubbled into the column from the bottom at a flow rate of 1 vvm ($L_{air} \cdot L_{reactor}{}^{-1} \cdot min^{-1}$). Aeration provided CO_2, between 260 and 380 ppm (Siegenthaler *et al*. 2005). A set of 6 fluorescent lamps (3 Sylvania Gro-Lux F57W and 3 Philips TLD 58W) providing 250 $\mu mole \cdot cm^{-2} \cdot s^{-1}$ were used as light source. The tests were conducted in a climatic chamber at controlled temperature 20 $\pm$ 1°C and a photoperiod 14:10 h light:dark. At the beginning of the experiment all reactors (except control reactor) (Table 2) were inoculated with *C. stigmatophora* in order to obtain an initial concentration of biomass between 50–100 $mg \cdot L^{-1}$. Each test media were tested in triplicates. Also, for each test media a control was included. The control was made in the same way that the test media, without the microalgae, to determine the evolution of nutrient removal.

Analytical Methods

Microalgae biomass was measured daily by optical density at 680 nm (OD_{680}). Samples were diluted by appropriate ratios to ensure that the measured values were in the range of 0.1–1.0. In order to convert the values of OD_{680} to biomass as dry weight, a linear regression equation was developed (Biomass ($mg \cdot L^{-1}$) = $0.0016 \cdot OD_{680} + 0.0612$; $R^2 = 0.998$). Biomass dry weight as suspended solids, was determined gravimetrically according to Standard Methods (APHA-AWWA-WPCF 1992). Samples from each flask were taken daily to determine phosphorus and nitrogen after filtration. Phosphorus was measured as orthophosphate (P-PO_4^{3-}) and nitrogen as the sum of nitrate and ammonium (ΣN: N-NO_3^- + N-NH_4^+). Analysis of nitrate, ammonium and phosphate where performed by colorimetric method following the Spectroquant® NOVA 60 spectrophotometer manual (Merck) according to standard methods (APHA-AWWA-WPCF 1992). Conductivity was measured by using a conductimeter GLP 32 by CRISON.

Once the cultures reached the stationary stage of growth the concentrations of four elements (C, N, H, and S) of biomass were determined using an element analyzer (Leco® CHNS 932). The dry biomass on algal solution was obtained by centrifugation (Centrifuge Mixtasel-BL Selecta®) at 6000 rpm for 15 min; the same procedure was repeated three times with the addition of distilled water. The pellet was dried in a vacuum freeze-dryer (LABCOCNO®) during 72 hours for subsequent measurements of cellular components.

Statistical Analysis

Data kinetic modeling was performed using the software STATISTICA 6.0 (Statsoft-company). The Quasi-Newton method estimation was used, with a convergence criterion

of 10–4. Confidence interval for $p \leq 0.05$ was calculated using the equation 1. Where, $\bar{X}$ is the mean, δ standard deviation and n the sample size.

RESULTS AND DISCUSSION

Biomass Growth

The temporal evolution of *C. stigmatophora* biomass in the different tests is represented with dots in Figure 1. The typical evolution of a batch culture was observed for all treatments except for SWW (Figure 1b): the period of physiological adjustment (lag phase) due to changes in nutrient or culture conditions in all tests was less than 25 hours; an accelerating growth phase where the cells have adjusted to the new environment and begin to grow and multiply; an exponential growth phase characterized by cell doubling and the biomass grow at a constant rate; and finally, a stationary phase where the biomass growth rate was practically zero as a result of nutrient depletion in the culture medium.

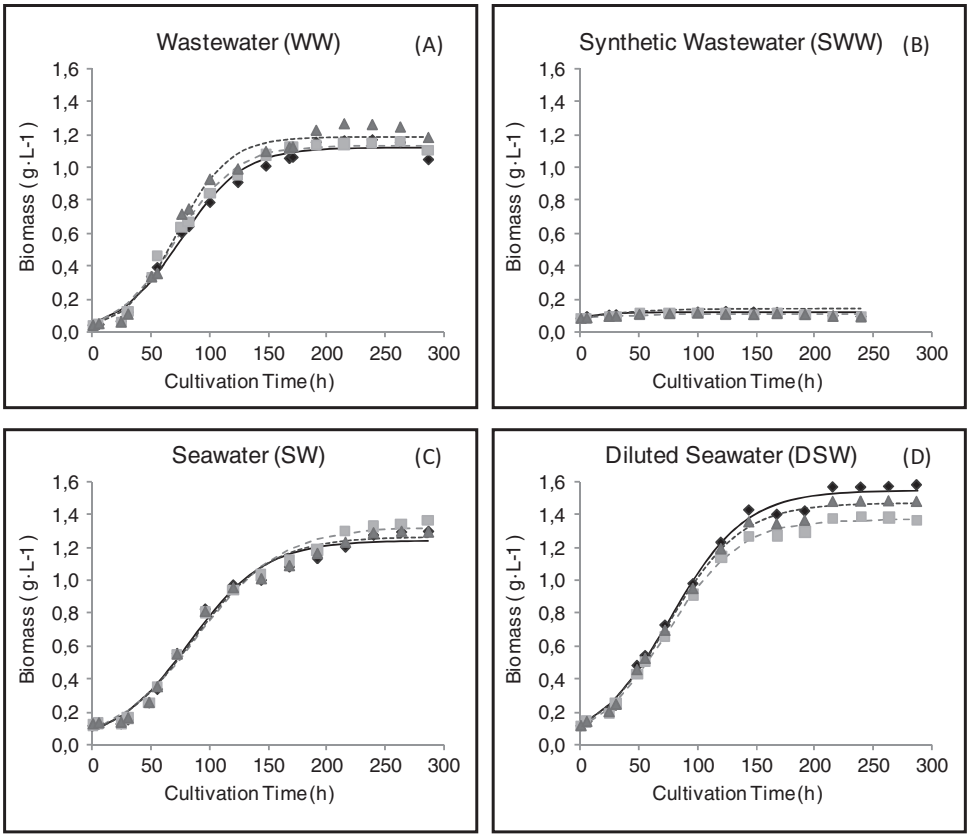

Figure 1 Algae growth curves (three replicates) in Wastewater WW (A), Synthetic Wastewater SWW (B), Seawater SW (C) and Diluted Seawater DSW (D). Experimental (dots) and predicted values (solid lines).

Table 3 Kinetic growth parameters (n = 3, p≤0.05) (± Confidence interval)

Culture medium	X_0 (gSS·L^{-1})	X_{max} (gSS·L^{-1})	μ_{max} (day^{-1})	*R^2
WW	0.052 ± 0.010	1.148 ± 0.041	1.008 ± 0.096	0.982
SWW	(+)	(+)	(+)	(+)
SW	0.093 ± 0.007	1.276 ± 0.048	0.696 ± 0.048	0.987
DSW	0.118 ± 0.008	1.461 ± 0.101	0.768 ± 0.024	0.995

*Lower value obtained from the three replicates.
(+)No growth was observed.

The Verlhust Logistic kinetic model (Verlhust 1838) was applied to the experimental results obtained in each test. That model is a substrate independent equation and can accurately describe the inhibition of biomass growth in different cultures conditions which occurs in many batch bioreactors (Gong and Lun 1996). According to the model, the microbial growth could also be expressed as a sinusoidal curve as described by equation 2, where, μ_{max} is the specific growth rate (day^{-1}), X_{max}, X_o and X the concentration of biomass (g SS·L^{-1}) at an operation time equal to infinite, zero and t respectively. By integrating equation 2 we get the equation 3. The maximum productivity (g SS·L^{-1}·day^{-1}) in steady state was calculated by using the maximum concentration achieved (X_{max}) and the specific growth rate (μ_{max}) by the equation 4.

Results of the modelization (Figure 1A,B,C, and D) showed a good adjustment between the experimental data and the predicted values obtained with Verlhust model (1838). In all the experiments the regression coefficient (R^2, p ≤ 0.05) was higher than 0.982 (Table 3) except the test SWW (Figure 1B) where inhibition of biomass growth was observed probably due to low salinity.

Table 3 shows that there are significant differences (p ≤ 0.05) for the maximum biomass concentration reached (X_{max}) in the different test media. X_{max} is higher in DSW than in SW and WW, which indicates that there is no relationship between X_{max} and the salinities of the media.

For the specific growth rates (μ_{max}) significant differences between cultures media were also observed (Table 3). The maximum specific growth rate was reached in WW, 1.008 ± 0.096 day^{-1}. The reason for this was probably due to the nitrogen source, in WW the nitrogen was mainly in the chemical species NH_4^+ while for SW and DSW the nitrogen was present in the most oxidized species NO_3^-. The microalgae often prefer N-NH_4^+ as nitrogen source rather other nitrogen species (described below).

A very important operational parameter is the productivity (Eq. 4). Productivity depends from one side the specific growth rate (μ_{max}) and from the other side the ability to achieve high concentrations (X_{max}), therefore the balance between those critical factors must be found. The results (Figure 2) showed that no significant difference in productivity between WW (1.146 ± 0.154 g SS·L^{-1}·day^{-1}) and the DSW (1.128 ± 0.048 g SS·L^{-1}·day^{-1}) reactors has been obtained.

On the other hand the productivity reached in SW was around 21% and 20% lower than in WW or DSW, the main reason for this behavior was because in SW the specific growth rate was the lowest (0.696 ± 0.048 day^{-1}) implying low growth rates compared with the other culture media (WW and DSW), which affects the final productivity achieved.

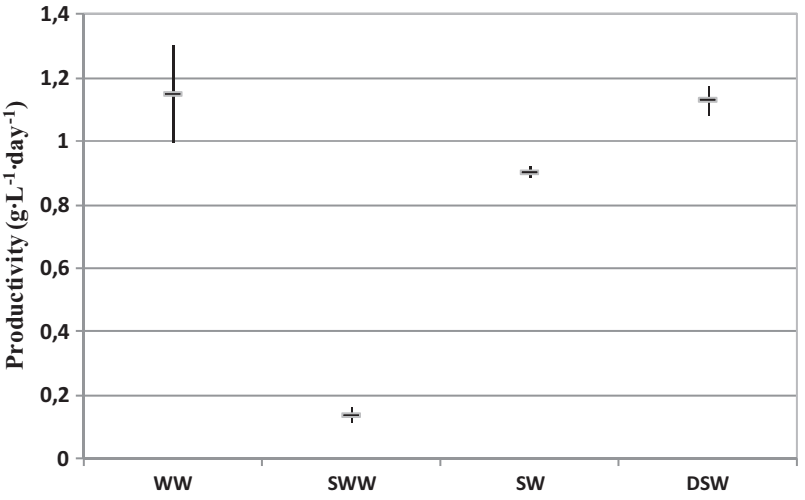

Figure 2 Productivity ($g \cdot L^{-1} \cdot day^{-1}$) obtained through Verhulst's model (1838) in Mean, maximum, and minimum values.

Nutrient Removal

Nitrogen removal. Nitrogen is mostly supplied as nitrate (NO_3^-), but ammonia (NH_4^+) and urea are also used with similar growth rates recorded (Kaplan *et al*. 1986). Ammonia nitrogen ($N\text{-}NH_4^+$) is often the preferred nitrogen source for microorganisms (Grobbelaar 2004). The main differences relative to nitrogen composition has been the ratio R_N: $N\text{-}NH_4^+/N\text{-}NO_3^-$ (Table 2). In WW and SWW R_N were 58.18 and 55.98 respectively while for the rest of tests, where the main nitrogen species was NO_3^-, R_N was less than 0.008.

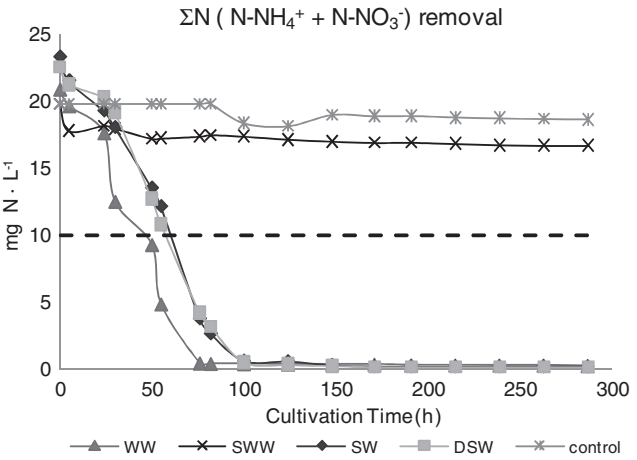

Figure 3 Evolution of ΣN ($N\text{-}NH_4^+ + N\text{-}NO_3^-$) concentration in test media (n = 3). Dotted line shows the most restrictive value for total nitrogen in the Directive 98/1565/CE (10 $mg \cdot L^{-1}$).

The evolution of ΣN under all conditions tested is depicted in Figure 3. The Figure shows that ΣN removal happens for biological processes because the controls did not present nitrogen decrease. In all reactors, ΣN was completely removed except in SWW where an inhibition of the biomass growth was observed (Figure 1b) and no significant ΣN removal occurred.

In order to compare nitrogen removal kinetics, an estimation of the time required to reach 10 mg $\Sigma N \cdot L^{-1}$ ($T_{10(N)}$) (most restrictive value in the Directive 98/1565/CE) has been done. In the WW test, $T_{10(N)}$ (45.15 hours) was lower than in SW and DSW 60.23 and 57.45 hours respectively. As the nutrient content (N and P) in all these reactors are similar, those differences in nitrogen removal kinetics is due to R_N. In WW the nitrogen source is almost 100% NH_4^+, nitrogen specie more easily assimilated by the algae (Grobbelaar 2004).

Phosphorous removal. The temporal evolution of P-PO_4^{3-} removal in all the tests show a pronounced fall in concentration during the first 5 h of testing (Figure 4). That initial drop in the P-PO_4^{3-} concentration implies rate removals up to 25% in the first 5 hours of experiment. According to different authors (Boyd and Musig 1981; Khummongkol *et al*. 1982; El Yousri 1995; Okada *et al*. 2004) the fall is attributed to two different processes: (1) adsorption to the surface of the reactor due to precipitation, and/or (2) adsorption to the microalgae cell wall and subsequent assimilation. As in the control reactors that initial fall was not observed (Figure 4), no removal due adsorption to the reactor surface or precipitation happened, so the initial high rate drop in P-PO_4^{3-} concentration is attributed to the adsorption to the microalgae cell wall.

In all the tests (except in SWW) the removal percentages were up to 99% at the end of the experiment. As in the case of nitrogen, the time required to reach de most restrictive P-PO_4^{3-} legal concentration $T_{1(P)}$ value for urban wastewater effluents (Directive 98/1565/CE) was determined. The results were similar for WW and DSW, 32.27 and 30 h respectively, being slightly lower in the case of SW (24.72 h).

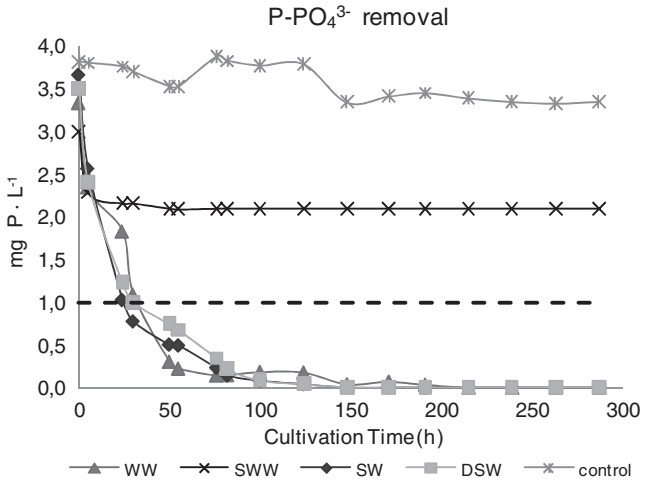

Figure 4 Evolution of P-PO_4^{3-} concentration in test media (n = 3). Dotted line shows the most restrictive value for total nitrogen in the Directive 98/1565/CE (1 $mg \cdot L^{-1}$).

Table 4 Elementary analysis of dried biomass obtained by Leco CHNS 932

Culture medium	C (%)	H (%)	N (%)	S (%)
WW	54.35 ± 0.13	8.89 ± 0.402	1.89 ± 0.09	0.37 ± 0.004
SWW	37.50 ± 1.00	8.25 ± 0.173	1.65 ± 0.12	0.65 ± 0.10
SW	47.86 ± 0.58	7.72 ± 0.485	2.52 ± 0.08	0.82 ± 0.02
DSW	54.54 ± 0.16	8.41 ± 0.049	2.53 ± 0.11	0.57 ± 0.03

Carbon Biofixation

Elementary analysis of the biomass is showed in Table 4. The calculated carbon percentage indicate a significant difference when the microalgae were cultivated in SW (47.86% ± 0.58 and in the culture media WW and DSW (54.35% ± 0.13 and 54.54% ± 0.16 respectively). So, an influence of the salinity in the biomass carbon content occurs and it could be due to the dependence of carbon dioxide solubility on **salted** water. In this sense, biomass cultured in higher salinity media present lower carbon content.

Results obtained from the biomass growth kinetic modeling (Table 3) and the carbon content analysis (Table 4) were used to obtain the maximum amount of CO_2 biofixed per reactor volume, R_{CO2max} (g $CO_2 \cdot L^{-1}$), the yield coefficient Y_{CO2} (g $CO_2 \cdot$g SS^{-1}) and the maximum CO_2 biofixation rate P_{vCO2} ($gCO_2 \cdot L^{-1} \cdot day^{-1}$) (Table 5).

R_{CO2max} fixed in DSW was 20.6 and 22.6% higher than in WW and SW media respectively. This difference is because R_{CO2max} is directly proportional to the maximum concentration of biomass reached (X_{max}) and the percentage of carbon in biomass, and DSW had the highest values X_{max} (1.461 ± 0.101 g $SS \cdot L^{-1}$) as well as carbon content (54.34%).

The results obtained for the yield coefficient ranging between 1.75 and 1.99 g $CO_2 \cdot g \cdot SS^{-1}$ (Table 5). Those values are similar to the obtained by other authors for different microalga species. Scragg *et al*. (2002) have reported that *Chlorella vulgaris* cultivated in Watanabe's medium and low nitrogen medium reached a yield coefficient of 1.875 g $CO_2 \cdot g \cdot SS^{-1}$ respectively. For *Chlorella kessleri*, De Morais and Costa (2007b) have reported a coefficient yield of 1.875 g $CO_2 \cdot g \cdot SS^{-1}$. *Scenedesmus obliquus* coefficient yield was of 1.931 and 1.771 g $CO_2 \cdot g \cdot SS^{-1}$ when it was cultivated in wastewater in summer and winter respectively (Gomez *et al*. 2005).

Table 5 Carbon dioxide biofixation kinetics parameters (n = 3)

Culture medium	[a]R_{CO2max} (g $CO_2 \cdot L^{-1}$)	[b]P_{vCO2} (g $CO_2 \cdot L^{-1} \cdot day^{-1}$)	[c]Y_{CO2} (g $CO_2 \cdot$g SS^{-1})
WW	2.335	2.324	1.992
SWW	1.654	2.088	1.375
SW	2.273	1.608	1.775
DSW	2.936	2.269	1.999

[a]R_{CO2max}: X_{max}.%C/100.
[b]P_{vCO2}: $\mu_{max} \cdot R_{CO2max}$.
[c]Y_{CO2}:100/%CO_2.

CONCLUSIONS

The marine microalgae *Chlorella stigmatophora* has excellent properties to be cultivated in urban wastewater effluents, with biomass productivities in wastewater (1.146 ± 0.154 g SS·L^{-1}·day^{-1}) higher than those obtained in nutrient enriched seawater 0.901 ± 0.020 g SS·L^{-1}·day^{-1}. In addition, the highest maximum specific growth rate was reached in the wastewater test (1.008 ± 0.096 day^{-1}) .This implies that the doubling time required for *C. stigmatophora* in wastewater is lower than in seawater and diluted seawater; This phenomenon is related to the composition of the culture medium.

Regarding the nutrient removal, the *C. stigmatophora* has a high potential for removing nitrogen and phosphorous from the urban wastewater even below the most restrictive levels established in the urban wastewater European normative (Directive 98/1565/CE). In WW test the $T_{10(N)}$ and $T_{1(P)}$ needed were 45.15 and 32.27 h respectively and at the end of the experiment the removal was 100% in both. Therefore according to the results obtained the *C. stigmatophora* preferentially assimilates N-NH_4^+ than N-NO_3^- as nitrogen source.

Finally, regarding the carbon dioxide biofixation capability, an influence of the salinity in the biomass carbon content occurs which could be due to the dependence of carbon dioxide solubility on salted water. Calculated carbon percentage indicates a significant difference when the microalgae were cultivated in SW (47.86% ± 0.58 and in the culture media WW and DSW (54.35% ± 0.13 and 54.54% ± 0.16 respectively).

ACKNOWLEDGMENT

This research was supported by the projects CTM2008-01249 financed by the Ministry of Science and Innovation (Spanish Government) and P08-TEP-03854 financed by the Council of Innovation, Science and Enterprise (Regional Government of Andalusia, Spain).

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APPENDIX

Equation 1:

$$\text{C.I} = \overline{\text{X}} \pm \left(\frac{\delta}{\sqrt{n}} \right) \tag{1}$$

Equation 2:

$$\frac{dX}{dt} = \mu_{\max\ X} \left(1 - \frac{X}{X_{\max}} \right) \tag{2}$$

Equation 3:

$$X = \frac{X_0 X_{\max} e^{\mu\ \max\ t}}{X_{\max} - X_o + X_o e^{\mu_{\max\ t}}} \tag{3}$$

Equation 4:

$$\text{P}_{\text{biomass max}} = \mu_{\max} \cdot \text{x}_{\max} \tag{4}$$